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BIOTECHNOLOGY and BIOENGINEERING

Expression of *merA*, *trxA*, *amoA*, and *hao* in Continuously Cultured *Nitrosomonas europaea* Cells Exposed to Cadmium Sulfate Additions

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Received 13 March 2009; revision received 19 June 2009; accepted 23 June 2009

Published online 2 July 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22454

ABSTRACT: The effects of $CdSO_4$ additions on the gene expressions of a mercury reductase, *merA*, an oxidative stress protein, *trxA*, the ammonia-monooxygenase enzyme (AMO), *amoA*, and the hydroxylamine oxidoreductase enzyme (HAO), *hao*, were examined in continuously cultured *N. europaea* cells. The reactor was fed 50 mM NH_4^+ and was operated for 78 days with a 6.9 days hydraulic retention time. Over this period, six successive batch additions of $CdSO_4$ were made with increasing maximum concentrations ranging from 1 to 60 μM Cd^{2+}. The expression of *merA* was highly correlated with the level of Cd^{2+} within the reactor ($Rs = 0.90$) with significant up-regulation measured at non-inhibitory Cd^{2+} concentrations. Cd^{2+} appears to target AMO specifically at lower concentrations and caused oxidative stress at higher concentrations, as indicated by the SOURs (specific oxygen uptake rates) and the up-regulation of *trxA*. Since Cd^{2+} inhibition is irreversible and *amoA* was up-regulated in response to Cd^{2+} inhibition, it is hypothesized that de novo synthesis of the AMO enzyme occurred and was responsible for the observed recovery in activity. Continuously cultured *N. europaea* cells were more resistant to Cd^{2+} inhibition than previously examined batch cultured cells due to the presence of Mg^{2+} and Ca^{2+} in the growth media, suggesting that Cd^{2+} enters the cell through Mg^{2+} and Ca^{2+} import channels. The up-regulation of *merA* during exposure to non-inhibitory Cd^{2+} levels indicates that *merA* is an excellent early warning signal for Cd^{2+} inhibition.

Biotechnol. Bioeng. 2009;104: 1004–1011.

KEYWORDS: *Nitrosomonas europaea*; chemostat; $CdSO_4$; *merA*; *amoA*; *trxA*

Introduction

The oxidation of ammonia to nitrite by ammonia oxidizing bacteria (AOB) in wastewater treatment plants (WWTPs) is often considered the most sensitive step in the nitrification process (U.S.EPA, 1993). Heavy metals, such as Cd^{2+}, enter WWTP influents primarily from industrial discharges and urban storm water runoff (Davis et al., 2001; Sorme, 2002; U.S.EPA, 2005; Wang, 2005) and are potent inhibitors of the nitrification process (Hu et al., 2003; U.S.EPA, 1993). Cd^{2+}, a representative divalent cation, has been shown to be highly inhibitory towards pure cultures of *Nitrosomonas europaea*, the model AOB (Chandran and Love, 2008; Park and Ely, 2008a).

N. europaea is an obligate chemolithoautotroph that derives all of its energy for growth solely from the oxidation of ammonia (NH_3) to nitrite (NO_2^-) (Wood, 1986). This two-step process utilizes ammonia-monooxygenase (AMO) to oxidize NH_3 to hydroxylamine (NH_2OH) and hydroxylamine oxidoreductase (HAO) to oxidize NH_2OH to NO_2^-. A net gain of 2 mol of electrons is generated for every mole of NH_3 oxidized, which are shuttled further down the electron transport chain for cell growth and maintenance (Arp et al., 2002). The relatively low number of electrons generated from ammonia oxidations leads to a slow doubling time of 8–12 h even under ideal conditions (Watson et al., 1981). The sensitivity of AOB to metal toxicity and inhibition combined with the slow growth rate can result in failures in the nitrification process. The early detection of Cd^{2+}-mediated nitrification inhibition in AOB may be useful in preventing and minimizing nitrification failures at WWTPs.

In previous batch studies, the expression of *merA*, a mercury resistance protein, and *trxA*, an oxidative stress protein, were found to be up-regulated in batch grown *N. europaea* cells and short time exposures to Cd^{2+} (Park and Ely, 2008a). This study builds on that work by determining

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Contract grant sponsor: National Science Foundation's Division of Bioengineering and Environmental Systems Genome-Enabled Environmental Sciences and Engineering Program

the suitability of *merA* and *trxA* as potential biomarkers (sentinel genes) for Cd^{2+}-induced inhibition under chemostat conditions that are more representative of those encountered in wastewater treatment.

Pure cultures of *N. europaea* were continuously grown in a bioreactor under chemostat conditions and exposed to increasing concentrations of Cd^{2+} added as successive pulses. The effect of Cd^{2+} on nitrification activity, in particular to AMO and HAO activity, was monitored throughout the experiment as was the expression of their encoding genes (*amoA* and *hao*, respectively) as well as the possible sentinel genes *merA* and *trxA*. Tests conducted in this manner allowed for several key observations including how long gene expressions were enhanced by the presence of Cd^{2+}, how quickly and at what concentrations the genes responded to the Cd^{2+} addition and were the gene responses correlated to the Cd^{2+} concentration in the system. Additional benefits to the chemostat experimental set-up included the detailed characterization of possible long-term inhibition and recovery mechanisms that would not likely be identifiable in a batch experiment set-up.

The current study also expands on previous work that examined the expression of *merA*, *amoA*, and *hao* of continuously cultured *N. europaea* cells that were exposed to pulse additions of $ZnCl_2$ (Radniecki et al., 2009). In that work, limited RNA sampling found that *merA* and *amoA* were increasingly up-regulated in response to increasing pulse additions of $ZnCl_2$ while *hao* expression did not change. In this work, the transient metabolic activities and transient gene expressions in response to changes in Cd^{2+} concentrations were studied in greater detail. Based on previous batch inhibition studies, where *merA* was found to be up-regulated in *N. europaea* cells exposed to Zn^{2+} and Cd^{2+} (Park and Ely, 2008a,b), it is hypothesized that Cd^{2+} may invoke transcriptional responses similar to Zn^{2+} in the continuously cultured *N. europaea* cells.

Methods

Organism and Cultivation

Details of the reactor operation are provided by Radniecki et al. (2009) and are briefly described here. *N. europaea* (ATCC 19718) was cultivated with a minimal media containing trace metals, HEPES buffer and 50 mM NH_4^+ in a 7 L water-jacketed Bioflo 110 bioreactor (New Brunswick Scientific, Edison, NJ) with a dilution rate of 0.14 day^{-1} to achieve a hydraulic residence time of 6.9 days. The bioreactor was operated in the dark at 30°C. The media was well mixed and the dissolved O_2 levels were kept at air saturation levels (8 mg/L) with the addition of filtered compressed air. The pH was maintained at 7.8 with the metered addition of 0.5 M Na_2CO_3.

Experimental Design (Bioreactor)

Once steady state was achieved within the bioreactor, as determined by protein, NO_2^- and NH_4^+ concentrations, $CdSO_4$, dissolved in DI water, was added as a spike addition and the protein, NO_2^-, NH_4^+, and Cd^{2+} concentrations along with ammonia-monooxygenase-specific oxygen uptake rate (AMO-SOUR) and hydroxylamine oxidoreductase-specific oxygen uptake rate (HAO-SOUR) were measured on cells harvested from the reactor. $CdSO_4$ was always added below the solubility limit so that Cd^{2+} ion would be in solution. Total RNA was extracted 1 h after the injection of the $CdSO_4$, the same length of time that batch *N. europaea* cells were exposed to $CdSO_4$ in previous experiments (Park and Ely, 2008a), and every 7 days (1 hydraulic residence time) thereafter. This process was repeated with 1, 5, 10, 20, 30, and 60 μM $CdSO_4$ spikes, over a period of operation from 11 to 78 days.

Analytical Procedures

Total NH_4^+, NO_2^-, and protein concentrations were measured in 3 mL aliquot samples from the reactor as previously described (Radniecki et al., 2009). Cd^{2+} concentrations were measured at 228.8 nm using a Varian Liberty 150 ICP Emission Spectrometer (Varian, Inc., Palo Alto, CA) using standard procedures (APHA, 1995).

AMO- and HAO-SOUR Measurements

Overall O_2 uptake rates of *N. europaea* are dominated by the oxidation of NH_3 to NH_2OH by AMO. The activity of AMO, AMO-SOUR, and the activity of HAO and other terminal electron acceptors, HAO-SOUR, were measured as described previously (Ely et al., 1995) using a water-jacketed glass cell fitted with a Clark microelectrode (Yellow Springs Instrument Co., Model # 5331, Yellow Springs, OH) attached to a YSI Model 5300 Biological Oxygen monitor (Yellow Springs Instrument Co.) and a flatbed strip chart recorder. Triplicate AMO- and HAO-SOUR measurements were made at each time point.

Reversibility Experiments

Five milliliters of continuously cultured *N. europaea* cells were withdrawn from the bioreactor, prior to the addition of $CdSO_4$, and placed into a 25 mL bottle and sealed with a Teflon coated cap. $(NH_4)_2SO_4$ and $CdSO_4$ were added to the bottles to achieve final concentrations of 2.5 mM and 20 μM, respectively. Bottles without the addition of $CdSO_4$ served as controls. The bottles were shaken at 250 rpm in the dark at 30°C for 15 min. After 15 min of exposure, the AMO-SOUR of the cells was measured, as described above. The cells were then centrifuged and washed five times in 30 mM HEPES buffer at pH 7.8 to remove Cd^{2+}. The washed cells were placed into fresh medium containing 2.5 mM $(NH_4)_2SO_4$ and AMO-SOURs were measured after 15 min of incubation to determine whether the Cd^{2+} dependent inhibition of ammonia oxidation was reversible. The tests were performed in triplicate.

Reverse Transcriptase-Quantitative PCR (RT-qPCR) Analysis

Gene Runner v. 3.00 (Hastings Software, Inc., Hastings on Hudson, NY) was used to generate RT-qPCR primers from *16S*, *merA*, *trxA*, *amoA*, and *hao* DNA sequences (Table I). The RT-qPCR primers were optimized for concentration (125 nM) and annealing temperature (55°C) via PCR to achieve high efficiency with only one product detected. Total RNA from cells, 200 mg wet-weight protein, was stabilized in Trizol (Invitrogen Co., Carlsbad, CA) and extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's instructions.

Total RNA concentrations and quality were determined spectrophotometrically using the Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) per manufacture's instructions. cDNA was generated from 1 μg of total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) per manufacturer's instruction and diluted 100-fold in TE buffer at pH 8.

RT-qPCR was carried out in triplicate on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the iQ SYBR Green Supermix kit (Bio-Rad) as described previously (Park and Ely, 2008b). The relative expression values for the reactions were determined using DART (Data Analysis for Real Time)-PCR analysis (Peirson et al., 2003) taking into account the efficiency of the reaction and normalizing the data to the amount of 16S RNA quantified in each reaction. For these experiments, the transcriptional responses of the bioreactor cells exposed to various Cd^{2+} concentrations were compared with the transcriptional responses of bioreactor cells that had achieved steady state prior to $CdSO_4$ addition.

Spearman Rank Correlations

The Spearman rank correlation (Rs) was used to measure the strength of correlation between *merA*, *amoA*, *hao*, *trxA*, AMO-SOUR, and HAO-SOUR with Cd^{2+} concentrations (Glantz, 2005). Linear regression analysis and ANOVA analysis of the regression were also performed to determine if the above variables were linearly and significantly correlated to Cd^{2+} concentrations.

Table I. RT-qPCR primers used in this study.

Primer name	Primer sequence
16S For	5′-GGCTTCACACGTAATACAATGG-3′
16S Rev	5′-CCTCACCCCAGTCATGACC-3′
merA For	5′-GCTTTATCAAGCTGGTCATC-3′
merA Rev	5′-ACATCCTTGTTGAAGGTCTG-3′
trxA For	5′-TTACTGGGCAGAATGGTGTG-3′
trxA Rev	5′-ACCTTGGTCGCCTCGATATT-3′
amoA For	5′-TGGCGACATACCTGTCACAT-3′
amoA Rev	5′-ACAATGCATCTTTGGCTTCC-3′
hao For	5′-CAAACTTGCCGAAATGAACC-3′
hao Rev	5′-GCTGGTGATGTTCTCTGCAA-3′

Trace Metal Protection

To determine the protective properties of trace metal nutrients on *N. europaea* to Cd^{2+} inhibition, tests with batch grown cells were conducted. *N. europaea* cells were cultured as previously described (Park and Ely, 2008a) and harvested at mid-exponential growth phase. Cells were washed with 30 mM HEPES buffer, pH 7.8, and suspended in 5 mL of media in a 25 mL bottle. The medium consisted of 30 mM HEPES buffer, pH 7.8, 2.5 mM $(NH_4)_2SO_4$, 5 μM $CdSO_4$ and a combination of the following growth medium salts; 10 μM KH_2PO_4, 270–2,250 μM $MgSO_4$, 270–2,250 μM $CaCl_2$, 18 μM $FeSO_4$, 1 μM $CuSO_4$, and 3.77 mM Na_2CO_3. Controls were run in the same medium without exposure to Cd^{2+}. Samples were shaken at 250 rpm at 30°C in the dark for 2 h and NO_2^- concentrations were determined every 0.5 h using the colorimetric assay as previously described (Radniecki et al., 2009), to determine the extent of inhibition.

Results

Chemostat Functionality

The chemostat was operated to achieve steady-state NO_2^- production (42 mM), protein level (11 mg/L), and NH_4^+ removal (below the detection limit of 0.1 mM) prior to Cd^{2+} pulse additions. Once steady state was achieved, pulse additions of Cd^{2+} were added and subsequently washed out of the reactor, consistent with a hydraulic residence time of 6.9 days (Fig. 1a and b). Based on the constant concentration of NO_2^-, protein and NH_4^+ observed, the functionality of the chemostat reactor was not affected by the addition of 1 or 5 μM Cd^{2+}. The addition of 10 and 20 μM Cd^{2+}, however, resulted in a decline in protein concentrations (9% and 39%, respectively) but did not result in a decline in NO_2^- concentrations or significant increase in NH_4^+.

The addition of a 30 μM pulse of $CdSO_4$ resulted in a 74% decrease in protein concentration (to 2.5 mg/L), a 57% decrease in NO_2^- concentration (to 28 mM), and an increase in NH_4^+ concentration up to 18 mM. The protein levels and NO_2^- concentrations began to increase and NH_4^+ concentrations decreased at about 54 days, 1 week after the Cd^{2+} pulse, at which time the Cd^{2+} concentration had decreased to 12 μM. Two weeks after the Cd^{2+} pulse addition (day 60) the NO_2^- concentration recovered to steady-state levels, the cell density was at 69% of steady-state levels, and NH_4^+ concentrations decreased to 5 mM. The subsequent addition of a 60 μM pulse of Cd^{2+} resulted in the washout of cells and NO_2^- within 14 days, while NH_4^+ concentrations increased towards the influent concentration.

merA Expression

Exposure of *N. europaea* to 5, 10, and 20 μM $CdSO_4$ resulted in 6-, 10-, and 20-fold up-regulation of *merA*, respectively

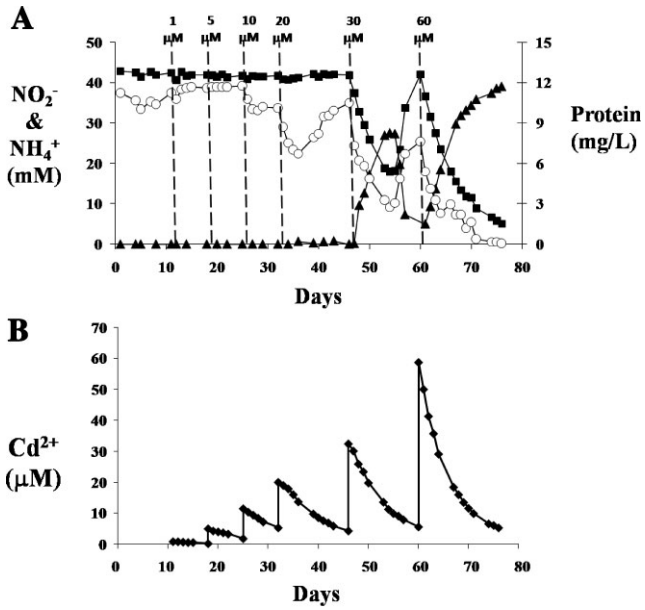

Figure 1. **A**: Physiological parameters of the chemostat monitored during the experiment. (○) Protein, (■) NO_2^-, and (▲) NH_4^+. Dashed lines represent when $CdSO_4$ was pulsed into the chemostat. **B**: The concentration of Cd^{2+} measured in the chemostat throughout the experiment.

(Fig. 2). The expression of *merA* was highly correlated with the levels of Cd^{2+} in the chemostat ($Rs = 0.90$), increasing with increased concentrations of Cd^{2+} and decreasing in expression as Cd^{2+} washed out of the system (Figs. 2 and 1b). The expression of *merA* was linearly correlated with Cd^{2+} concentrations of 30 μM and below ($R^2 = 0.83$, $P = 5\ E-07$) and was the most sensitive of the genes evaluated ($m = 0.93$-fold up-regulation/μm Cd^{2+}) (Table II). A weaker linear correlation was obtained when the data from the 60 μM Cd^{2+} pulse was included ($R^2 = 0.59$, $P = 0.0001$). The lower correlation resulted from the expression of *merA*

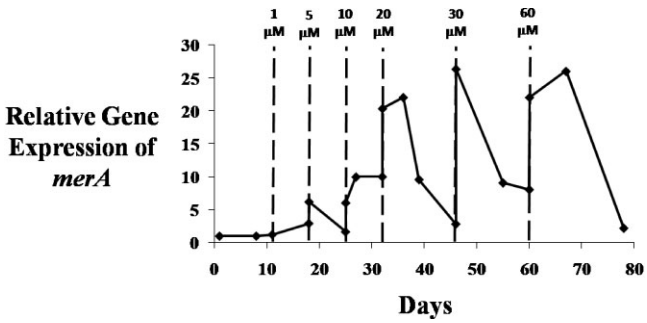

Figure 2. The relative fold change of *merA* in continuously cultured *N. europaea* cells exposed to pulsed Cd^{2+} additions. Dashed lines indicate when Cd^{2+} was pulsed into the bioreactor.

being about the same level at both 30 and 60 μM Cd^{2+} (Fig. 2). The leveling off of *merA* expression at high Cd^{2+} concentrations may have resulted from a shortage of energy available for gene expression due to NO_2^- production inhibition. Another possibility is that the cells have reached a biological maximum gene expression rate for *merA* in this system.

AMO and HAO Activity

To examine how Cd^{2+} was affecting *N. europaea* at the metabolic enzyme level, AMO and HAO-SOURs were measured throughout the experiment (Fig. 3a and b). The AMO-SOURs were more sensitive to Cd^{2+} inhibition than chemostat concentrations of NO_2^- and NH_4^+. Decreased AMO-SOURs were observed at Cd^{2+} concentrations as low at 10 μM (Fig. 3a) whereas NO_2^- disappearance and NH_4^+ appearance in the chemostat did not occur until Cd^{2+} levels reached 30 μM (Fig. 1a). AMO-SOURs decreased with each increase in Cd^{2+} concentration reaching a maximum decrease in activity of 82% after the pulse addition of 30 μM Cd^{2+}. AMO-SOURs were negatively correlated with Cd^{2+} concentrations and the linear correlation was weak (Table II).

HAO-SOURs were not correlated with Cd^{2+} concentrations and there was not a linear relationship (Table II). HAO-SOURs were not adversely affected at Cd^{2+} concentrations up to 20 μM. However, decreases in HAO-SOURs followed by a recovery of HAO-SOURs above steady-state levels did occur upon exposure to Cd^{2+} concentrations of 30 μM and greater.

Cd^{2+} Inhibition Reversibility

Batch tests with continuously cultured *N. europaea* cells were performed to evaluate the reversibility of Cd^{2+} inhibition. Exposure of continuously cultured *N. europaea* cells to 20 μM Cd^{2+} resulted in 58% inhibition in nitrite productions rates. After removing the Cd^{2+}, these cells showed 70% inhibition in nitrite production rates (data not shown). These experiments demonstrated that Cd^{2+}-induced inhibition is not reversible.

amoA and *hao* Expression

The relative expression of *amoA* increased over steady-state levels with each pulse addition of Cd^{2+} and reached a maximum relative expression of 7.6-fold up-regulation after exposure to 60 μM Cd^{2+} (Fig. 4a). Similar to *merA*, the expression of *amoA* was correlated with Cd^{2+} concentrations ($Rs = 0.72$) but was less sensitive ($m = 0.22$) than *merA*. Additionally, the linearity of the expression of *amoA* was not statistically significant with respect to Cd^{2+} concentrations (Table II), making *amoA* expression a less

Table II. The linearity (R^2) and correlation (Rs) of *merA*, *amoA*, *hao*, and *trxA* gene expression and AMO- and HAO-SOURs with respect to Cd^{2+} concentration.

	All concentrations					Up to 30 μM Cd^{2+}				
Gene or SOUR	Rs	R^2	*P*-value	*m*	*b*	Rs	R^2	*P*-value	*m*	*b*
merA	0.90	0.59	0.0001	0.48	4.59	0.88	0.83	5 E − 07	0.93	1.45
amoA	0.72	0.17	0.08	0.05	2.75	0.64	0.66	0.07	0.22	1.48
hao	0.47	0.09	0.22	0.02	1.81	0.24	0.01	0.75	0.01	1.90
trxA	0.82	0.32	0.01	0.09	2.05	0.80	0.26	0.05	0.12	1.67
AMO-SOUR	−0.46	0.38	0.005	−0.02	1.21	−0.45	0.42	0.01	−0.03	1.32
HAO-SOUR	0.10	0.08	0.23	−0.01	1.48	0.41	0.01	0.90	0.002	1.38

The linear regression equations are represented by *m* (the slope) and *b* (the *y*-intersect). The *P*-value indicates the statistical significance of the calculated R^2. The left hand columns include data from the pulse addition of 60 μM Cd^{2+} ($n = 20$) while the right hand columns exclude this data ($n = 16$).

attractive indicator of Cd^{2+} inhibition than *merA* expression.

The general trend in *hao* expression was a very slight up-regulation upon exposure to all concentrations of Cd^{2+} tested followed by a slight decrease in *hao* expression as Cd^{2+} washed out of the chemostat (Fig. 4b). The expression of *hao* was not very sensitive to and weakly correlated with Cd^{2+} concentrations (Table II). However, unlike *merA* and similar to *amoA*, there was not a statistically significant linear relationship of *hao* expression with Cd^{2+} concentrations (Table II).

Oxidative Stress and *trxA* Expression

The expression of *trxA* was monitored over the course of the experiment as a possible indicator of oxidative stress on *N. europaea* cells. The relative gene expression of *trxA* increased with each pulse addition of Cd^{2+} and generally decreased as Cd^{2+} washed out of the chemostat and reached a maximum eightfold up-regulation after the pulsed additions of 30 and 60 μM of Cd^{2+} (Fig. 5). Similar to *merA* and *amoA*, the expression of *trxA* was positively correlated with Cd^{2+} concentrations. However, the expression of *trxA* is not as linear with or as sensitive to Cd^{2+} concentrations as *merA* but is more linear and sensitive than *amoA* (Table II).

Trace Metal Protection

To investigate the relationship between trace metals and Cd^{2+} inhibition sensitivity, separate batch experiments

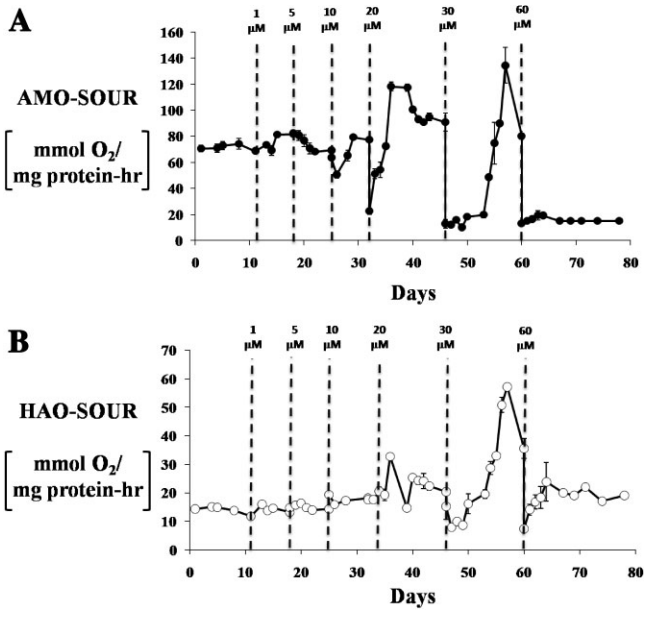

Figure 3. The (**A**) AMO-SOUR (●) and (**B**) HAO-SOUR (○) of continuously cultured cells exposed to Cd^{2+} additions. The dashed line indicates when Cd^{2+} was pulsed into the bioreactor. Error bars represent 95% confidence intervals.

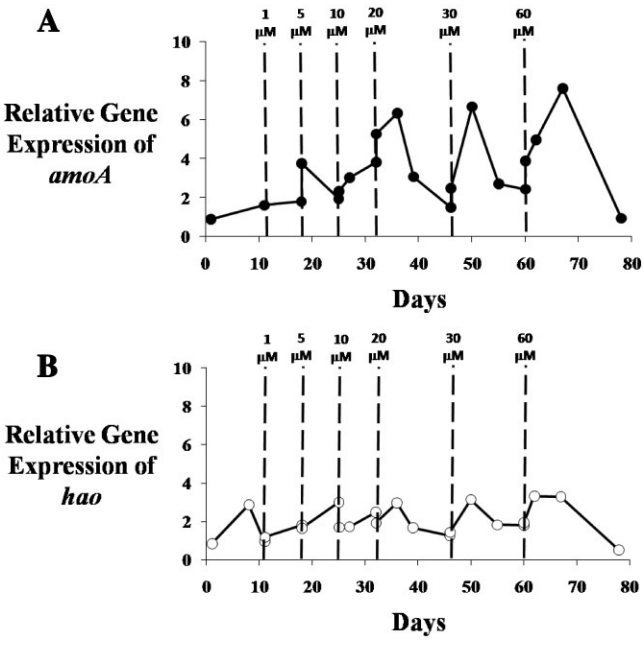

Figure 4. The relative fold change of *amoA* (**A**); and *hao* (**B**) in continuously cultured *N. europaea* cells exposed to pulsed $CdSO_4$ additions. Dashed lines indicate when Cd^{2+} was pulsed into the bioreactor.

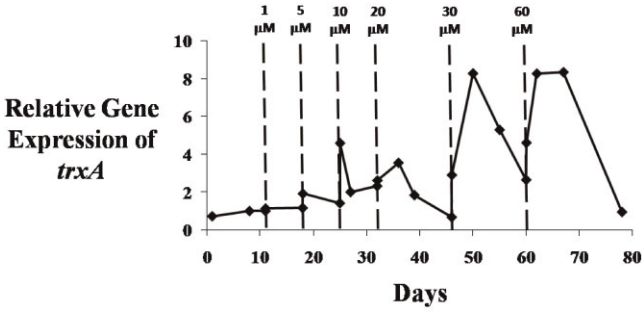

Figure 5. The relative fold change of *trxA* in continuously cultured *N. europaea* cells exposed to pulsed Cd^{2+} additions. Dashed lines indicate when Cd^{2+} was pulsed into the bioreactor.

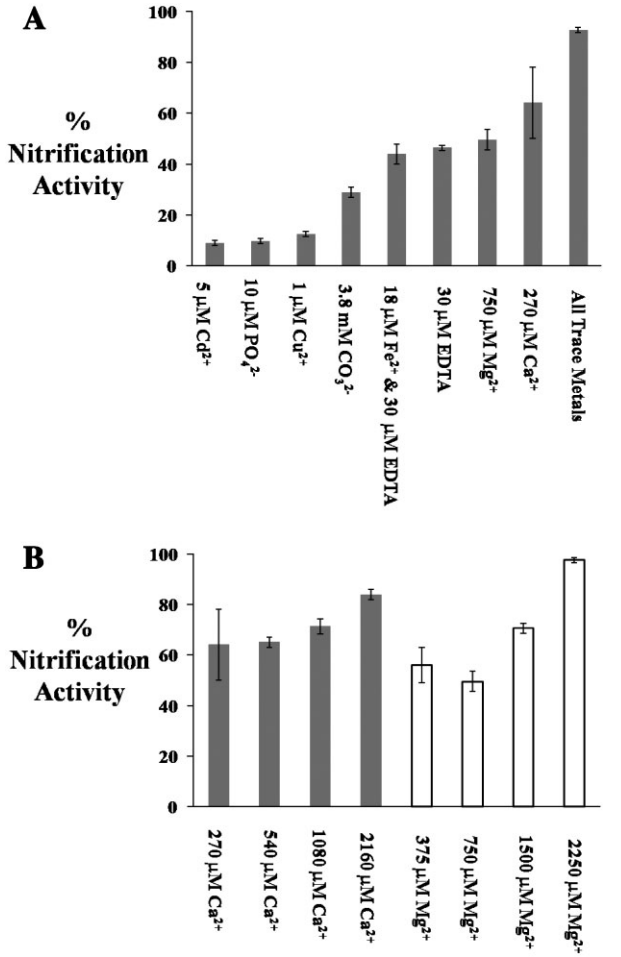

Figure 6. The effect of media composition (**A**) and specifically Ca^{2+} and Mg^{2+} concentrations (**B**) on the nitrite production inhibition of *N. europaea* caused in the presence of 5 µM Cd^{2+}. *N. europaea* cells were placed in 30 mM HEPES, pH 7.8, 5 mM NH_4^+ and various trace elements as shown above and incubated at 30°C for 120 min with the nitrification activity measured and compared to the control every 30 min. Error bars represent 95% confidence intervals.

were conducted with batch grown cells exposed to 5 µM Cd^{2+}, 5 mM NH_4^+ and the various trace metals at concentrations used in the chemostat experiments. The 5 µM Cd^{2+} concentration was chosen as the model Cd^{2+} concentration due to the severe nitrification inhibition it induced (~90%) in the absence of trace metals (Fig. 6a). The presence of trace metals was found to play an important role in the protection of *N. europaea* cells from Cd^{2+} inhibition (Fig. 6a). The divalent cations Ca^{2+} and Mg^{2+} along with the metal chelator EDTA provided the most protection for the cells exposed to 5 µM Cd^{2+} with cells retaining 64%, 50%, and 46% of their initial activity, respectively. The summation of all trace metals provided nearly 100% protection from exposure to 5 µM Cd^{2+}.

To further investigate the role of Ca^{2+} and Mg^{2+} in the protection of *N. europaea* from Cd^{2+} inhibition, batch cells were exposed to 5 µM Cd^{2+} and various concentrations of Ca^{2+} or Mg^{2+}. The addition of higher concentrations of Ca^{2+} or Mg^{2+} resulted in increased protection of *N. europaea* from Cd^{2+} inhibition (Fig. 6b). Cells exposed to 5 µM Cd^{2+} and 2,160 µM Ca^{2+} or 2,250 µM Mg^{2+} retained 84% and 98% of their activity, respectively, while cells not exposed to Ca^{2+} or Mg^{2+} only retained 10% of their activity.

Discussion

One of the major goals of this study was to evaluate the response of potential Cd^{2+} sentinel genes under continuously cultured conditions. In previous batch experiments, *merA* was found to be up-regulated 297-fold in *N. europaea* cells exposed to 1 µM Cd^{2+} (Park and Ely, 2008a) and was thus chosen as a potential sentinel gene for this work. The gene expression of *merA* was found to be the most sensitive of the genes tested with respect to Cd^{2+} concentrations (Table II). The up-regulation of *merA* was observed at sub-inhibitory Cd^{2+} concentrations (5–20 µM) with regards to nitrite production rates (Figs. 1a and 2) and indicates that *merA* is an excellent early-warning Cd^{2+} sentinel gene. In addition, the significant linear correlation between *merA* transcript levels and Cd^{2+} concentrations within the reactor (Table II) suggests that a nitrification inhibition biosensor based on *merA* expression would not only identify Cd^{2+} as an inhibitor but may also be semi-quantitative.

While the MerA protein, encoded by *merA*, is responsible for reducing the toxic Hg^{2+} to a more non-toxic Hg^0 (Nies, 1999) it is currently unclear what role the MerA protein plays in response to non-redox active metals such as Cd^{2+}. However, *merA* is a part of a larger mercury resistance operon which also includes several transport proteins (Bruins et al., 2000) and many bacteria exhibit resistance to Cd^{2+} inhibition through cadmium efflux (Nies, 1999). It may be the case the *N. europaea* is actively up-regulating the mercury resistance operon transport proteins in a generic heavy metal stress response and *merA* is being up-regulated because it belongs to the same operon. Further research is

needed to clarify the role of the mercury resistance operon in response to non-redox active heavy metals, such as Cd^{2+}.

The activity of AMO (AMO-SOURs) and HAO, along with other terminal electron acceptors, (HAO-SOURs) were monitored throughout the experiment to determine how Cd^{2+} affected *N. europaea*'s metabolic enzymes and the electron transport chain. AMO-SOURs proved to be a more sensitive measure of nitrification inhibition in the chemostat than measuring NO_2^-, NH_4^+, and protein levels within the reactor. Pulse Cd^{2+} additions of 20 μM or less resulted in decreased AMO-SOURs but not HAO-SOURs (Fig. 3a and b). This indicates that at low Cd^{2+} concentrations Cd^{2+} specifically targets the AMO enzyme.

After the initial inhibition, AMO-SOUR activities generally rose to above steady-state levels during the course of the next few days, followed by a return to steady-state activity levels, generally within 2 weeks of the Cd^{2+} pulse. The recovery of AMO-SOUR activity was not likely due the removal of Cd^{2+} as Cd^{2+} inhibition is irreversible and the recovery begins with inhibitory Cd^{2+} concentrations still present within the reactor. The creation of new cells may play a role in the recovery, but is not likely the primary source of the recovery due to the relatively short time-span of recovery (1–3 days) and the long doubling time of the continuously cultured *N. europaea* cells (5 days).

The up-regulation of *amoA* that was observed may offer some insight into how *N. europaea* cells regained their AMO activity. The expression of *amoA* remained high while AMO-SOUR activity was below steady-state levels and decreased to steady-state levels when AMO-SOUR activity spiked. Since AMO-SOUR activity is normalized to cell concentrations, this suggests that the spike in AMO-SOUR activity above steady-state levels was due to an increased concentration of AMO within the cells. As *amoA* expression returned to steady-state levels as Cd^{2+} washed out of the reactor, the AMO-SOUR activity also returned to steady-state levels, suggesting that the AMO concentrations within the cell had resumed steady-state levels as well. This dynamic pattern of *amoA* over-expression and AMO-SOUR activity spikes followed by a return of steady state levels of *amoA* expression and AMO-SOUR activity, along with the irreversibility of Cd^{2+} inhibition that targets the AMO enzyme directly, suggests that *N. europaea* cells recovered from Cd^{2+} inhibition through the de novo synthesis of AMO enzymes.

The exact mechanism of AMO inhibition by Cd^{2+}, a non-redox active divalent cation, is not known. One possibility is that Cd^{2+} is replacing Cu^{2+} or Fe^{3+}, two redox active multivalent cations, in the active site of AMO. Heavy metals such as Cd^{2+} have been shown to replace biologically important redox active metal cores in enzymes (Nies, 1999; Stohs, 1995) and Zn^{2+}, another non-redox active divalent cation, has been suggested to replace Cu^{2+} in the AMO enzyme of *Nitrosococcus mobilis* and *N. europaea* (Radniecki and Ely, 2008; Radniecki et al., 2009). Another possible inhibition mechanism is that Cd^{2+} is binding to AMO at a non-active site and that the binding is altering the shape and function of the active site resulting in AMO inhibition. Further research is needed to elucidate the exact mechanism of Cd^{2+}-based AMO inhibition.

With Cd^{2+} pulse additions of 30 and 60 μM, HAO-SOURs decreased as well as AMO-SOURs (Fig. 3a and b). This decrease in HAO-SOURs suggests that a more global stress, such as oxidative stress, is being exerted on the cells. The eightfold up-regulation of *trxA*, an oxidative stress protein, at 30 and 60 μM Cd^{2+} indicates that oxidative stress is occurring (Fig. 5). Previous research has shown that Cd^{2+} exposure resulted in oxidative stress on *N. europaea* cells (Chandran and Love, 2008). However, it is unlikely that Cd^{2+}, a non-redox active heavy metal, is directly creating oxidative stress. One possibility is that Cd^{2+} is replacing Fe^{3+} and Cu^{2+} in biological enzymes, such as AMO as discussed above, and that these liberated cations are undergoing Fenton reactions resulting in the formation of hydroxyl radicals that cause oxidative stress (Stohs, 1995).

Similar to AMO-SOURs, HAO-SOURs recovered as Cd^{2+} washed out of the reactor. However, the expression of *hao* changed very moderately over the experiment (Fig. 3) and it is thus unlikely that de novo synthesis of HAO is responsible for the recovery of HAO-SOURs after exposure to 30 and 60 μM Cd^{2+}.

A curious observation noticed throughout the experiment was that the continuously cultured *N. europaea* cells were found to have an increased resistance to Cd^{2+} inhibition compared to previous batch tests with *N. europaea* cells (Park and Ely, 2008a). Additionally, in previous batch studies with pure cultures of *N. europaea*, 1 h of exposure to 1 μM $CdSO_4$ resulted in a 197-fold increase in *merA* (Park and Ely, 2008a). In this study with continuously cultured *N. europaea* cells, *merA* was up-regulated a maximum of 26-fold above steady-state levels upon exposure to 30 μM Cd^{2+} (Fig. 2). A similar difference in *merA* gene expression between batch and continuously cultured cells was observed in *N. europaea* cells exposed to Zn^{2+} (Park and Ely, 2008b; Radniecki et al., 2009).

The primary difference between the two experiments was the composition of the test media. The chemostat media contained trace metals whereas the media in the previously conducted batch experiments did not. A systematic test of the protective properties of the various trace metals used in the chemostat experiment revealed that an excess in Ca^{2+} and Mg^{2+} concentrations protected *N. europaea* from Cd^{2+} inhibition (Fig. 6a and b). Similar protection of Ca^{2+} and Mg^{2+} was observed in a previous chemostat study with the exposure of *N. europaea* to Zn^{2+}(Radniecki et al., 2009).

The up-take of non-essential divalent cations, such as Cd^{2+}, into cells have been previously shown to occur through Mg^{2+}, Ca^{2+}, and general cation transport systems (Clemens, 1998; Nies, 1989, 1999). This suggests that it may be possible that Cd^{2+} is competing with Ca^{2+} and Mg^{2+} for entrance into the cell through either Mg^{2+} transporters, such as *mgtE*, Ca^{2+} transporters, or more general divalent cation transporters, such as *czc* (Chain et al., 2003). However, future research needs to test a wider range

of Ca^{2+}, Mg^{2+}, and Cd^{2+} concentrations to confirm if a concentration dependent competition into the cell is occurring.

These observations indicate the importance of media composition on the sensitivity of *N. europaea* to Cd^{2+} inhibition. It also indicates a potential strategy of protecting WWTPs from nitrification failure through the addition of protective cations in response to the presence of Cd^{2+} or Zn^{2+}.

Conclusion

This study has shown that the expression of *merA* is a sensitive, robust and semi-quantitative measure of Cd^{2+}-induced nitrification inhibition and has great potential for use in nitrification inhibition biosensors. Additionally, Cd^{2+} employs a dual-inhibition mechanism of targeting the AMO enzyme directly and inducing a more global oxidative stress at higher concentrations. Under the chemostat conditions of these tests, *N. europaea* appeared to recover from Cd^{2+} inhibition through the de novo synthesis of AMO. Finally, elevated Ca^{2+} and Mg^{2+} concentrations were able to protect *N. europaea* cells from Cd^{2+} inhibition. While the Cd^{2+} concentrations used in this study were in the micromolar range and further research is needed to determine if excess Ca^{2+} and Mg^{2+} can protect *N. europaea* cells from higher Cd^{2+} concentrations, the addition of excess Ca^{2+} and Mg^{2+} may be a useful tool for WWTP operators experiencing nitrification failure due to heavy metal inhibition.

We thank Luis Sayavedra-Soto for the *N. europaea* culture, Dan Arp and Peter Bottomley on providing constructive input on the work, Mohammad Azizian for help with the analytical techniques used, Sun Hwa Park and Roger Ely for supplying the *merA* RT-qPCR primers and early access to the results of their research findings, the Oregon State University Center for Genome Research and Biocomputing for RT-qPCR assistance and Yuri Gorby and the Pacific Northwest National Labs for providing valuable input on continuous culturing techniques. Funding was provided by a grant from the National Science Foundation's Division of Bioengineering and Environmental Systems Genome-Enabled Environmental Sciences and Engineering Program.

References

APHA. 1995. Standard methods for the examination of water and wastewater. 3120B. Coupled Plasma (ICP) Method.

Arp DJ, Sayavedra-Soto LA, Hommes NG. 2002. Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. Arch Microbiol 178(4):250–255.

Bruins MR, Kapil S, Oehme FW. 2000. Microbial resistance to metals in the environment. Ecotoxicol Environ Saf 45:198–2007.

Chain P, Lamerdin J, Larimer F, Regala W, Lao V, Land M, Hauser L, Hooper A, Klotz M, Norton J, Sayavedro-Soto L, Arciero D, Hommes N, Whittaker M, Arp D. 2003. Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. J Bacteriol 185(21):6496–6496.

Chandran K, Love NG. 2008. Physiological state, growth mode, and oxidative stress play a role in Cd(II)-mediated inhibition of *Nitrosomonas europaea* 19718. Appl Environ Microbiol 74(8):2447–2453.

Clemens S. 1998. The plant cDNA LCT1 mediates the uptake of calcium and cadmium in yeast. Proc Natl Acad Sci USA 95(20):12043–12048.

Davis AP, Shokouhian M, Ni SB. 2001. Loading estimates of lead, copper, cadmium, and zinc in urban runoff from specific sources. Chemosphere 44(5):997–1009.

Ely RL, Hyman MR, Arp DJ, Guenther RB, Williamson KJ. 1995. A cometabolic kinetics model incorporating enzyme inhibition, inactivation, and recovery 2. Trichloroethylene degradation experiments. Biotechnol Bioeng 46(3):232–245.

Glantz SA. 2005. Primer of biostatistics. New York: McGraw-Hill Professional.

Hu ZQ, Chandran K, Grasso D, Smets BF. 2003. Impact of metal sorption and internalization on nitrification inhibition. Environ Sci Technol 37(4):728–734.

Nies DH. 1989. Metal-ion uptake by a plasmid-free metal-sensitive *Alcaligenes eutrophus* strain. J Bacteriol 171(7):4073–4075.

Nies DH. 1999. Microbial heavy-metal resistance. Appl Microbiol Biotechnol 51(6):730–750.

Park S, Ely RL. 2008a. Candidate stress genes of *Nitrosomonas europaea* for monitoring inhibition of nitrification by heavy metals. Appl Environ Microbiol 74(17):5475–5482.

Park S, Ely RL. 2008b. Genome-wide transcriptional responses of *Nitrosomonas europaea* to zinc. Arch Microbiol 189(6):541–548.

Peirson SN, Butler JN, Foster RG. 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Res 31(14):e73.

Radniecki TS, Ely RL. 2008. Zinc chloride inhibition of *Nitrosococcus mobilis*. Biotechnol Bioeng 99(5):1085–1095.

Radniecki TS, Semprini L, Dolan ME. 2009. Expression of *merA*, *amoA* and *hao* in continuous cultured *Nitrosomonas europaea* exposed to zinc chloride. Biotechnol Bioeng 102(2):546–553.

Sorme L. 2002. Sources of heavy metals in urban wastewater in Stockholm. Sci Total Environ 298(1–3):131–145.

Stohs SJ. 1995. Oxidative mechanisms in the toxicity of metal-ions. Free Radic Biol Med 18(2):321–336.

U.S.EPA. 1993. Process Design Manual: Nitrogen control. Washington, DC: U.S. Environmental Protection Agency. Report nr 625/R-93/010.

EPA US. 2005. Toxicological review of zinc and compounds. Washington, DC: U.S. Environmental Protection Agency, Report nr 7440-66-6.

Wang C. 2005. Total concentrations and fractions of Cd, Cr, Pb, Cu, Ni and Zn in sewage sludge from municipal and industrial wastewater treatment plants. J Hazard Mater 119(1–3):245–249.

Watson SW, Valois FW, Waterbury JB. 1981. The Family Nitrobacteraceae. In: Starr MP, Stolp H, Truper HG, Balows A, Schlegel HG, editors. The prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. New York: Springer-Verlag, p 1005–1022.

Wood PM. 1986. Nitrification as a bacterial energy source. In: Prosser JI, editor. Nitrification. Washington, DC: Society for General Microbiology (IRL Press), p 39–62.